

In re Appl. No. 09/893,878

**REMARKS**

The purpose of this amendment is to conform the specification to the sequence listing and correct typographical errors. Almost all of the amendments were made previously in 08/009,319, now USP 5,403,484, see amendment of April 27, 1993, and/or in 08/993,776, see amendment of April 5, 2000 and January 25, 2001. The present amendment is lengthier merely because of the rule change which requires that amendments show the whole affected paragraph(s) in rewritten form.

Note that the correction of "GGGSL" to "GSSSL" at pp. 217-18 is based on p281, L31-32.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Respectfully submitted,

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**Enclosures**

- substitute pages 466, 466a, 467-71
- substitute pages with yellow highlight to show insertions
- marked-up version of the amended text

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

The paragraph beginning at page 34, lines 24-27, has been amended as follows:

BPTI	Bovine pancreatic trypsin inhibitor, identical to aprotinin (Merck Index, entry 784, p.119 <u>(SEQ ID NO:44)</u> )
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The paragraph on page 38, lines 10-18, has been amended as follows:

A candidate IPBD should meet the following criteria:

- 1) a domain exists that will remain stable under the conditions of its intended use (the domain may comprise the entire protein that will be inserted, e.g. BPTI (SEQ ID NO:44),  $\alpha$ -conotoxin GI, or CMTI-III),
- 2) knowledge of the amino acid sequence is obtainable, and
- 3) a molecule is obtainable having specific and high affinity for the IPBD, AfM (IPBD).

The paragraph on pages 50-51, lines 18-34 and 1-8, has been amended as follows:

Polypeptides of this size, however, have disadvantages as binding molecules. According to Olivera et al. (OLIV90a): "Peptides in this size range normally equilibrate among many conformations (in order to have a fixed conformation, proteins generally have to be much larger)." Specific binding of a peptide to a target molecule requires the peptide to take up one conformation that is complementary to the binding site. For a decapeptide with three isoenergetic conformations (e.g.,  $\beta$  strand,  $\alpha$  helix, and reverse turn) at each residue, there are about  $6 \cdot 10^4$  possible overall conformations. Assuming these conformations to be equi-probable for the unconstrained decapeptide, if only one of the possible conformations bound to the binding site, then the affinity of the peptide for the

target ~~would be~~ expected to be about  $6 \cdot 10^4$  higher if it could be constrained to that single effective conformation. Thus, the unconstrained decapeptide, relative to a decapeptide constrained to the correct conformation, would be expected to exhibit lower affinity. It would also exhibit lower specificity, since one of the other conformations of the unconstrained decapeptide might be one which bound tightly to a material other than the intended target. By way of corollary, it could have less resistance to degradation by proteases, since it would be more likely to provide a binding site for the protease.

Please replace the paragraph on page 52, lines 2-14, with the following rewritten paragraph:

In vitro, disulfide bridges can form spontaneously in polypeptides as a result of air oxidation. Matters are more complicated in vivo. Very few intracellular proteins have disulfide bridges, probably because a strong reducing environment is maintained by the glutathione systems. Disulfide bridges are common in proteins that travel or operate in ~~intracellular extracellular~~ spaces, such as snake venoms and other toxins (e.g., conotoxins, charybdotoxin, bacterial enterotoxins), peptide hormones, digestive enzymes, complement proteins, immunoglobulins, lysozymes, protease inhibitors (BPTI and its homologues, CMTI-III (Cucurbita maxima trypsin inhibitor III) and its homologues, hirudin, etc.) and milk proteins.

The paragraph on page 58, lines 9-26, has been amended as follows:

Metal Finger Mini-Proteins. The mini-proteins of the present invention are not limited to those crosslinked by disulfide bonds. Another important class of mini-proteins are analogues of finger proteins. Finger proteins are characterized by finger structures in which a metal ion is

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coordinated by two Cys and two His residues, forming a tetrahedral arrangement around it. The metal ion is most often zinc(II), but may be iron, copper, cobalt, etc. The "finger" has the consensus sequence (Phe or Tyr)-(1 AA)-Cys-(2-4 Aas)-Cys-(3 Aas)-Phe-(5 Aas)-Leu-(2 Aas)-His-(3 Aas)-His-(5 Aas) (~~SEQ ID NOs:1,2,3,4,5,6~~) (BERG88; GIBS88). While finger proteins typically contain many repeats of the finger motif, it is known that a single finger will fold in the presence of zinc ions (FRAN87; PARR88). There is some dispute as to whether two fingers are necessary for binding to DNA. The present invention encompasses mini-proteins with either one or two fingers. It is to be understood that the target need not be a nucleic acid.

The paragraph on pages 63-64, lines 31-33 and 1-12, has been amended as follows:

Sauer and colleagues (PAKU86, ~~REID88-REID88a~~), and Caruthers and colleagues (EISE85) have shown that some residues on the polypeptide chain are more important than others in determining the 3D structure of a protein. The 3D structure is essentially unaffected by the identity of the amino acids at some loci; at the other loci only one or a few types of amino acid is allowed. In most cases, loci where wide variety is allowed have the amino acids side group directed toward the solvent. Loci where limited variety is allowed frequently have the side group directed toward other parts of the protein. Thus substitutions of amino acids that are exposed to solvent are less likely to affect the 3D structure than are substitutions at internal loci. (See also SCHU79, p169-171 and CREI84, p239-245, 314-315).

The paragraph on page 79, lines 1-6, has been amended as follows:

1) SNT which encodes the set [L,P,H,R,V,A,D,G]: a) one acidic (D) and one basic (R), b) both aliphatic (L,V) and

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aromatic hydrophobics (H), c) large (L,R,H) and small (G,A) side groups, d) ~~ridged rigid~~ (P) and flexible (G) amino acids, e) each amino acid encoded once.

The paragraph on page 79, lines 14-17, has been amended as follows:

4) VNT which encodes the set [L,P,H,R,I,T,N,S,V,A,D,G]:  
a) one acidic, one basic, b) all classes: charged, neutral hydrophilic, hydrophobic, ~~ridged rigid~~ and flexible, etc., c) each amino acid encoded once.

The paragraph on page 84, lines 1-8, has been amended as follows:

We vary a1, c1, g1, a1 and c2 and then calculate t1, g2, and t2. Initially, variation is in steps of 5%. Once an approximately optimum distribution of nucleotides is determined, the region is further explored with steps of 1%. The logic of this program is shown in Table 9. The optimum distribution (the ~~"fxs"~~ "gfk" codon) is shown in Table 10A and yields DNA molecules encoding each type amino acid with the abundance shown.

The paragraph on page 84, lines 9-17, has been amended as follows:

Note that this chemistry encodes all twenty amino acids, with acidic and basic amino acids being equiprobable, and the most favored amino acids (serine) is encoded only 2.454 times as often as the least favored amino acid (tryptophan). The ~~"fxs"~~ "gfk" vg codon improves sampling most for peptides containing several of the amino acids [F,Y,C,W,H,Q,I,M,N,K,D,E] for which NNK or NNS provide only one codon. Its sampling advantages are most pronounced when the library is relatively small.

The paragraph on page 85, lines 4-14, has been amended as

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follows:

If we reduce the prevalence of SER by reducing  $T_1$ ,  $C_2$ ,  $A_1$ , and  $G_2$ , relative to other bases, then we will also reduce the prevalence of PHE, TYR, CYS, PRO, THR, ALA, ARG, GLY, ILE, and ASN. The prevalence of LEU, HIS, VAL, and ASP will rise. If we assume that  $T_1$ ,  $C_2$ ,  $A_1$ , and  $G_2$  are all lowered to the same extent and that  $C_1$ ,  $G_1$ ,  $T_2$ , and  $A_2$  are increased by the same amount, we can compute a shift that makes the prevalence of SER equal the prevalences of LEU, HIS, VAL, and ASP. The ~~decrease decreases~~ in ~~each of~~ PHE, TYR, CYS, PRO, THR, ALA, ARG, GLY, ILE, and ASN ~~is are~~ not equal; CYS and THR are reduced more than the others.

The paragraph on page 89, lines 10-23, has been amended as follows:

Several of the preferred simple or complex variegated codons encode a set of amino acids which includes cysteine. This means that some of the encoded binding domains will feature one or more cysteines in addition to the invariant disulfide-bonded cysteines. For example, at each NNT-encoded position, there is a one in sixteen chance of obtaining cystein. If six codons are so varied, the fraction of domains containing additional cysteines is 0.33. Odd numbers of cysteines can lead to complications, see Perry and Wetzel (PERR84, ~~PERR86~~). On the other hand, many disulfide-containing proteins contain cysteines that do not form disulfides, e.g. trypsin. The possibility of unpaired cysteines can be dealt with in several ways:

The paragraph on page 94, lines 17-31, has been amended as follows:

In the first round, we assume that the parental protein has no known affinity for the target material. For example, consider the parental mini-protein, similar to that discussed in Example 11, having the structure  $X_1$ - $C_2$ - $X_3$ - $X_4$ - $X_5$ - $X_6$ - $C_7$ - $X_8$  ~~(SEQ~~

ID NO: 7) in which C<sub>2</sub> and C<sub>7</sub> form a disulfide bond. Introduction of extra cysteines may cause alternative structures to form which might be disadvantageous. Accidental cysteines at positions 4 or 5 are thought to be potentially more troublesome than at the other positions. We adopt the pattern of variegation: X<sub>1</sub>:NNT, X<sub>3</sub>:NNT, X<sub>4</sub>:NNG, X<sub>5</sub>:NNG, X<sub>6</sub>:NNT, and X<sub>8</sub>:NNT, so that cysteine can not occur at positions 4 and 5 (DNA sequence NNT.TGT.NNT.NNG.NNG.NNT.TGT.NNT has SEQ ID NO: 89). (Table 131 shows the number of different amino acids expected in libraries prepared with DNA variegated in this way and comprising different numbers of independent transformants.)

The paragraph on page 109, lines 11-30, has been amended as follows:

Optionally, DNA encoding a flexible linker of one to 10 amino acids is introduced between the ipbd gene fragment and the Pf3 coat-protein gene. Optionally, DNA encoding the recognition site for a specific protease, such as tissue plasminogen activator or blood clotting Factor Xa, is introduced between the ipbd gene fragment and the Pf3 coat-protein gene. Amino acids that form the recognition site for a specific protease may also serve the function of a flexible linker. This tripartite gene is introduced into Pf3 so that it does not interfere with expression of any Pf3 genes. To reduce the possibility of genetic recombination, part (3) is designed to have numerous silent mutations relative to the wild-type gene. Once the signal sequence is cleaved off, the IPBD is in the periplasm and the mature coat protein acts as an anchor and phage-assembly signal. It matters not that this fusion protein comes to rest anchored in the lipid bilayer by a route different from the route followed by the wild-type coat protein.

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The paragraph on page 138, lines 5-23, has been amended as follows:

Consider a mini-protein embedded in LamB. For example, insertion of DNA encoding  $G_1NXCX_5XXXCX_{10}SG_{12}$  (SEQ ID NO:8) between codons 153 and 154 of lamB is likely to lead to a wide variety of LamB derivatives being expressed on the surface of E. coli cells.  $G_1$ ,  $N_2$ ,  $S_{11}$ , and  $G_{12}$  are supplied to allow the mini-protein sufficient orientational freedom that is can interact optimally with the target. Using affinity enrichment (involving, for example, FACS via a fluorescently labeled target, perhaps through several rounds of enrichment), we might obtain a strain (named, for example, BEST) that expresses a particular LamB derivative that shows high affinity for the predetermined target. An octapeptide having the sequence of the inserted residues 3 through 10 from BEST is likely to have an affinity and specificity similar to that observed in BEST because the octapeptide has an internal structure that keeps the amino acids in a conformation that is quite similar in the LamB derivative and in the isolated mini-protein.

The paragraph on page 155, lines 16-20, has been amended as follows:

```
residue :      17  18  19  20  21  22  23  24  25
5'  g|gcc|gcG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|fxk|fxk|
g|gcc|gcG|GTA|CCG|ATG|CTG|TCT|TTT|GCT|qfk|qfk|
      26  27  28  29  30
fxk|TTC|TGT|CTC|GAG|cgc|ccg|cga|
qfk|TTC|TGT|CTC|GAG|cgc|ccg|cga| 3' olig#14
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The paragraph on page 155, lines 23-27, has been amended as follows:

```
residue :      17  18  19  20  21  22  23  24  25  26
5'  g gcc gcc GTA CCG ATG CTG TCT TTT GCT fxx fxx fxx
qcc gcc GTA CCG ATG CTG TCT TTT GCT qfk qfk qfk
      26a 26b  27  28  29  30
fxx fxx TTC TGT CTC GAG cgc ccg cga
qfk qfk TTC TGT CTC GAG cgc ccg cga 3' olig#14a
```

The paragraph on page 155, lines 30-34, has been amended as follows:

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residue : 17  18  19  20  21  22  23  24  25  26
5' g gcc gcc GTA CCG ATG CTG TCT TTT GCT fxx fxx fxx
5' g gcc gcc GTA CCG ATG CTG TCT TTT GCT qfk qfk qfk
      26a 26b 26c 26d  27  28  29  30
fxx fxx fxx fxx TTC TGT CTC GAG cgc ccg cga
qfk qfk qfk qfk TTC TGT CTC GAG cgc ccg cga 3' olig#14b
```

The paragraph on pages 155-156, lines 40-44 and 1-6, has been amended as follows:

where ~~fg~~ is a mixture of (0.26 T, 0.18 C, 0.26 A, and 0.30 G), ~~xf~~ is a mixture of (0.22 T, 0.16 C, 0.40 A, and 0.22 G), and k is a mixture of equal parts of T and G. The bases shown in lower case at either end are spacers and are not incorporated into the cloned gene. One of the variegated oligo-nts and the primer are combined in equimolar amounts and annealed. The ds DNA is completed with all four (nt)TPs and Klenow fragment. The resulting dsDNA and RF pLG7 are cut with both KpnI and XhoI, purified, mixed, and ligated. We selected a transformed clone that, when induced with IPTG, binds AHTrp or trp.

The paragraph on page 161, lines 2-10, has been amended as follows:

The target materials may be organic macromolecules, such

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as polypeptides, lipids, polynucleic acids, and polysaccharides, but are not so limited. Almost any molecule that is stable in aqueous solvent may be used as a target. The following list of possible targets is given as illustration and not as limitation. The categories are not strictly mutually exclusive. The omission of any category is not to be construed to imply that said category is unsuitable as a target. Merck Index refers to the Eleventh Edition.

The paragraph on page 163, lines 23-25, has been amended as follows:

2) ds DNA :      5'-CCGTCGAATCCGC-3' (SEQ ID NO:90)  
                  3'-GGCATTTAGGCG-5' (SEQ ID NO:91)  
                  (Note mismatch)

The paragraph on page 163, lines 26-27, has been amended as follows:

3) ss DNA :      5'-CGTAACCTCGTCATTA-3'  
                  (No hair pin) (SEQ ID NO:92)

The paragraph on page 163, lines 28-30, has been amended as follows:

4) ss DNA :      5'-CCGTAGGT  
                  3'-GGCATCCA  
                  (Note hair pin) (SEQ ID NO:93)

The paragraph on page 163, lines 31-33, has been amended as follows:

5) dsDNA with cohesive ends:  
                  5'-CACGGCTATTACGGT-3' (SEQ ID NO:94)  
                  3'-CCGATAATGCCA-5' (SEQ ID NO:95)

The paragraph on page 216, lines 23-25, has been amended

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as follows:

--M K K S - rest of VIII  
ACT.TCC.TC.ATG.AAA.AAG.TCT. (SEQ ID NOs:96 and 97)  
rest of XI - T S S stop--

On page 216 after line 26, insert a new paragraph as follows:

(The amino acid sequence MKKS has SEQ ID NO:9)

The paragraph on page 216, lines 29-31, has been amended as follows:

--(L) K K S - rest of VIII  
ACT.TCC.AG.CTG.AAA.AAG.TCT. (SEQ ID NOs:98 and 99)  
rest of XI -T S S stop--

The paragraph on pages 217-218, lines 25-33 and 1-6, has been amended as follows:

With regard to the 'EGGGS linker' (SEQ ID NO:10) extensions of the domain interface, individual phage stocks predicted to contain one or more 5-amino-acid unit extensions were analyzed in a similar fashion. The migration of the extended fusion proteins were readily distinguishable from the parent fusion protein when viewed by western analysis or silver staining. Those clones analyzed in more detail included M13.3X4 (which contains a single inverted EGGGS (SEQ ID NO:10) linker with a predicted amino acid sequence of ~~GGGSL~~ GSSSL (SEQ ID NO: 16)), M13.3X7 (which contains a correctly oriented linker with a predicted amino acid sequence of EGGGS (SEQ ID NO:10)), M13.3X11 (which contains 3 linkers with an inversion and a predicted amino acid sequence for the extension of EGGSGSSSLGSSSL (SEQ ID NO:11)) and M13.3Xd which contains an extension consisting of at least 5 linkers or 25 amino acids.

The paragraph on page 218, lines 21-31, has been amended

as follows:

The assay is detailed in ~~section EE-Example II~~, but principally involves the addition of purified anti-BPTI IgG (from the serum of BPTI injected rabbits) to a known titer of phage. Following incubation, protein A-agarose beads are added to bind the IgG and left to incubate overnight. The IgG-protein A beads and any bound phage are removed by centrifugation followed by a retitering of the supernatant to determine any loss of phage. The phage bound to the beads can be acid eluted and titered also. Appropriate controls are included in the assay, such as a wild type phage stock (M13mp18) and IgG purified from normal rabbit pre-immune serum.

The paragraph on pages 220-221, lines 30-34 and 1-10, has been amended as follows:

To take this analysis one step further, a comparison of phage binding to active and inactivated trypsin is shown in Table 145. The control phage, M13mp18 and BPTI-III MK, demonstrated binding similar to that detailed in Example III. Note that the relative binding is enhanced with trypsin due to the apparent marked reduction in the non-specific binding of the wild type phage to the active protease. M13.3X7 and M13.3X11, which both contain 'EGGGS' linker ~~(SEQ ID NO:10)~~ extensions at the domain interface, bound to anhydrotrypsin and trypsin in a manner similar to BPTI-IIIMK phage. The binding, relative to non-display phage, was approximately 100 fold higher in the anhydrotrypsin binding assay and at least 1000 fold higher in the trypsin binding assay. The binding of another 'EGGGS' linker variant (M13.3Xd) was similar to that of M13.3X7.

The paragraph on page 221, lines 11-27, has been amended as follows:

To demonstrate the specificity of binding the assays were

repeated with human neutrophil elastase (HNE) beads and compared to that seen with trypsin beads Table 146. BPTI has a very high affinity for trypsin and a low affinity for HNE, hence the BPTI display phage should reflect these affinities when used in binding assays with these beads. The negative and positive controls for trypsin binding were as already described above while an additional positive control for the HNE beads, BPTI(K15L,-MGNG)-III MA (see Example III) was included. The results, shown in Table 146, confirmed this prediction. M13MB48, M13.3X7 and M13.3X11 phage demonstrated good binding to trypsin, relative to wild type phage and the HNE control (BPTI(K15L,MGNG)-III MA) (The amino acid sequence MGNG has SEQ ID NO:12; BPTI(.....,MGNG) denotes a homologue of BPTI having M<sub>39</sub>, G<sub>40</sub>, N<sub>41</sub>, G<sub>42</sub> where ..... may indicate other alterations.), being comparable to BPTI-IIIMK phage. Conversely, poor binding occurred when HNE beads were used, with the exception of the HNE positive control phage.

The paragraph on pages 232-233, lines 24-33 and 1-20, has been amended as follows:

Fifty  $\mu$ l of BPTI-III MK phage (identified as MK-BPTI is ~~USPN 07/487,063 Example II~~) ( $3.7 \cdot 10^{11}$  pfu/ml) in either 50 mM Tris, pH 7.5, 150 mM NaCl, 1.0 mg/ml BSA (TBS/BSA) buffer or 50 mM sodium citrate, pH 6.5, 150 mM NaCl, 1.0 mg/ml BSA (CBS/BSA) buffer were added to 10  $\mu$ l of a 25% slurry of immobilized trypsin (Pierce Chemical Co., Rockford, IL) also in TBS/BSA or CBS/BSA. As a control, 50  $\mu$ l MK phage ( $9.3 \cdot 10^{12}$  pfu/ml) were added to 10  $\mu$ l of a 25% slurry of immobilized trypsin in either TBS/BSA or CBS/BSA buffer. The infectivity of BPTI-III MK phage is 25-fold lower than that of MK phage; thus the conditions chosen above ensure that an approximately equivalent number of phage particles are added to the trypsin beads. After 3 hours of mixing on a Labquake shaker (Labindustries Inc., Berkeley, CA) 0.5 ml of either TBS/BSA or CBS/BSA was added where appropriate to the samples. Beads

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were washed for 5 min and recovered by centrifugation for 30 sec. The supernatant was removed and 0.5 ml of TBS/0.1% Tween-20 was added. The beads were mixed for 5 minutes on the shaker and recovered by centrifugation as above. The supernatant was removed and the beads were washed an additional five times with TBS/0.1% Tween-20 as described above. Finally, the beads were resuspended in 0.5 ml of elution buffer (0.1 M HCl containing 1.0 mg/ml BSA adjusted to pH 2.2 with glycine), mixed for 5 minutes and recovered by centrifugation. The supernatant fraction was removed and neutralized by the addition of 130  $\mu$ l of 1 M Tris, pH 8.0. Aliquots of the neutralized elution sample were diluted in LB broth and titered for plaque-forming units on a lawn of cells.

The paragraph on page 242, lines 6-27, has been amended as follows:

A comparison of the structures of BI-8e and BPTI-(K15L) reveals the presence of three positively charged residues at positions 39, 41, and 42 of BPTI which are absent in BI-8e. These hydrophilic and highly charged residues of BPTI are displayed on a loop which underlies the loop containing the P1 residue and is connected to it via a disulfide bridge. Residues within the underlying loop (in particular residue 39) participate in the interaction of BPTI with the surface of trypsin near the catalytic pocket (BLOW72) and may contribute significantly to the tenacious binding of BPTI to trypsin. However, these hydrophilic residues might hamper the docking of BPTI variants with HNE. In support of this hypothesis, BI-8e displays a high affinity for HNE and contains no charged residues in the region spanning residues 39-42. Hence residues 39 through 42 of wild type BPTI were replaced with the corresponding residues of the human homologue of Bi-8e. We anticipated that a BPTI derivative containing the MET-GLY-ASN-GLY (MGNG) sequence (SEQ ID NO:12) would exhibit a higher affinity for HNE than corresponding derivatives which retain

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the sequence of wild type BPTI at residues 39-42.

The paragraph on pages 242-243, lines 28-34 and 1-19, has been amended as follows:

A double stranded oligonucleotide with AccI and EagI compatible ends was designed to introduce the desired alteration of residues 39 to 42 via cassette mutagenesis. Codon 45 was altered to create a new XmnI site, unique in the structure of the BPTI gene, which could be used to screen for mutants. This alteration at codon 45 does not alter the encoded amino-acid sequences. BPTI-IIIMA Rf DNA was digested with AccI. Two oligonucleotides (CYSB and CYST) corresponding to the bottom and top strands of the mutagenic DNA were annealed and ligated to the AccI digested BPTI-III MA Rf DNA. The sample was digested with BglII and the 2.1 kb BglII/EagI fragment was purified. BPTI-III MA Rf was also digested with BglII and EagI and the 6.0 kb fragment was isolated and ligated to the 2.1 kb BglII/EagI fragment described above. Ligation samples were used to transfect competent cells which were plated to permit the formation of plaques on a lawn of cells. Phage derived from plaques were probed with a radioactively labelled oligonucleotide (CYSB) using the Dot Blot Procedure. Positive clones were identified by autoradiography of the Nytran membrane after washing at high stringency conditions. Rf DNA was prepared from Ap<sup>R</sup> cultures containing fusion phage which hybridized to the CYSB probe. Restriction enzyme analysis and DNA sequencing confirmed that codons 39-42 of BPTI had been altered. The Rf DNA was designated BPTI(MGNG)-III MA (The amino acid sequence MGNG has SEQ ID NO:12; BPTI(.....MGNG)-III MA denotes a strain of M13 that displays BPTI(.....MGNG) fused to the gIII protein and that carries the bla gene that confers Ap<sup>R</sup>.).

The paragraph on pages 250-251, lines 9-35 and 1-5, has been amended as follows:

The MYMUT oligonucleotide permits the substitution of 5 hydrophobic residues (PHE, LEU, ILE, VAL, and MET via a DTS codon (D = approximately equimolar A, T, and G; S = approximately equimolar C and G)) for LYS<sub>15</sub>. Replacement of LYS<sub>15</sub> in BPTI with aliphatic hydrophobic residues via semi-synthesis has provided proteins having higher affinity for HNE than BPTI (~~TANN77-TANK77~~, JERI74a,b, WENZ80, TSCH86, BECK88b). At position 16, either GLY or ALA are permitted (GST codon). This is in keeping with the predominance of these two residues at the corresponding positions in a variety of BPTI homologues (CREI87). The variegation scheme at position 17 is identical to that at 15. Limited data is available on the relative contribution of this residue to the interaction of BPTI homologues with HNE. A variety of hydrophobic residues at position 17 was included with the anticipation that they would enhance the docking of a BPTI variant with HNE. Finally at positions 18 and 19, 4 (PHE, SER, THR, and ILE via a WYC codon (W - approximately equimolar A and T; Y = approximately equimolar T and C)) and 5 (SER, PRO, THR, LYS, GLN, and stop via an HMA codon (H = approximately equimolar A, C, and T; M = approximately equimolar A and C)) different amino acids respectively are encoded. These different amino acid residues are found in the corresponding positions of BPTI homologues that are known to bind to HNE (CREI87). Although the amino acids included in the PEPI library were chosen because there was some indication that they might facilitate binding to HNE, it was not and is not possible to predict which combination of these amino acids will lead to high affinity for HNE. The mutagenic oligonucleotide MYMUT was synthesized by Genetic Design Inc. (Houston, Texas).

The paragraph on pages 257-258, lines 10-34 and 1-7, has been amended as follows:

d) Characterization of Selected Fusion Phage

The pH 2.0 fraction from the third passage of the mini-



library was titered and plaques were obtained on a lawn of cells. Twenty plaques were picked at random and phage derived from plaques were probed with the CYSB oligonucleotide via the Dot Blot Procedure. Autoradiography of the filter revealed that all 20 samples gave a positive hybridization signal indicating that fusion phage were present and the DNA encoding residues 35 to 47 of BPTI(MGNG) is contained within the recombinant M13 genomes. Rd DNA was prepared for the 20 clones and initial dideoxy sequencing revealed that 12 clones were identical. This sequence was designated EpiNE $\alpha$  (SEQ ID NO:45 and SEQ ID NO:108) (Table 207). No DNA sequence changes were observed apart from the planned variegation. Hence the cassette mutagenesis procedure preserved the context of the planned variegation of the pepi gene. The Dot Blot Procedure was employed to probe all 20 selected clones from the pH 2.0 fraction from the third passage of the mini-library with an oligonucleotide homologous to the sequence of EpiNE $\alpha$ . Following high stringency washing, autoradiography revealed that all 20 selected clones were identical in the P1 region. Furthermore dot blot analysis revealed that of the 28 different phage samples pooled to create the mini-library, only one contained the EpiNE $\alpha$  sequence. Hence in just three passes of the mini-library over HNE beads, 1 out of 28 input fusion phage was selected for and appears as a pure population in the lowest pH fraction from the third passage of the library. That the EpiNE $\alpha$  phage elute at pH 3.5 while BPTI(K15V,R17L)-III MA phage elute at a higher pH strongly suggests that the EpiNE $\alpha$  protein has a significantly higher affinity than BPTI(K15V,R17L) for HNE.

The paragraph on pages 259-260, lines 25-34 and 1-19, has been amended as follows:

b) Characterization of Selected Clones

The pH 2.0 fraction from the third enrichment cycle of MYMUT library was titered on a lawn of cells. Twenty plaques

were picked at random. Rf DNA was prepared for each of the clones and fusion phage were collected by PEG precipitation. Clonally pure populations of fusion phage in TBS/BSA were prepared and characterized with respect to their affinity for immobilized HNE. pH elution profiles were obtained to determine the stringency of the conditions required to elute bound fusion phage from the HNE matrix. Figure 9 illustrates the pH profiles obtained for EpiNe clones 1 (SEQ ID NO:51), 3 (SEQ ID NO:46) and 7 (SEQ ID NO:48). The pH profiles for all 3 clones exhibit a peak centered on pH 3.5. Unlike the pH profile obtained for the third passage of the MYMUT library, no minor peak centered on pH 4.5 is evident. This is consistent with the clonal purity of the selected EpiNE phage utilized to generate the profiles. The elution peaks are not symmetrical and a prominent trailing edge on the low pH side. In all probability, the 10 minute elution period employed is inadequate to remove bound fusion phage at the low pH conditions. EpiNe clones 1 through 8 have the following characteristics: five clones (identified as EpiNE1 (SEQ ID NO:51), EpiNE3 (SEQ ID NO:46), EpiNE5 (SEQ ID NO:52), EpiNE6 (SEQ ID NO:47), and EpiNE7 (SEQ ID NO:48)) display very similar pH profiles centered on pH 3.5. The remaining 3 clones elute in the pH 3.5 to 4.0 range. There remains some diversity amongst the 20 randomly chosen clones obtained from the pH 2.0 fraction of the third passage of the MYMUT library and these clones might exhibit different affinities for HNE.

The paragraph on pages 264-265, lines 32-34 and 1-4, has been amended as follows:

Individual phage clones were picked, grown and analyzed for binding to Cat G beads. Figure 13 shows the binding and pH profiles for the individual Cat G binding clones (designated EpiC variants). All clones exhibited minor peaks, superimposed upon a gradual fall in bound phage, at pH elutions of 5 (clones 1 (SEQ ID NOs:54 and 117), 8 (SEQ ID

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NOs:56 and 119), 10 (SEQ ID NOs:57 and 120) and 11 (SEQ ID NOs: 54 and 117)) or pH 4.5 (clone 7 (SEQ ID NOs:55 and 118)).

The paragraph on page 265, lines 5-21, has been amended as follows:

DNA sequencing of the EpiC clones, shown in Table 209 (SEQ ID NOs:54 through 58 and 117 through 121), demonstrated that the clones selected for binding to Cat G beads represented a distinct subset of the available sequences in the MYMUT library and a cluster of sequences different from that obtained when enriched with HNE beads. The P1 residue in the EpiC mutants is predominantly MET, with one example of PHE, while in BPTI it is LYS and in the EpiNE variants it is either VAL or LEU. In the EpiC mutants residue 16 is predominantly ALA with one example of GLY and residue 17 is PHE, ILE or LEU. Interestingly residues 16 and 17 appear to pair off by complementary size, at least in this small sample. The small GLY residue pairs with the bulky PHE while the relatively larger ALA residue pairs with the less bulky LEU and ILE. The majority of the available residues in the MYMUT library for positions 18 and 19 are represented in the EpiC variants.

The paragraph on page 266, lines 4-14, has been amended as follows:

In Example IV, we described engineered protease inhibitors EpiNE1 through EpiNE8 (SEQ ID NOs:46 through 53 and 109-116) that were obtained by affinity selection. Modeling of the structure of the BPTI-Trypsin complex (Brookhaven Protein Data Bank entry 1TPA) indicates that the EpiNE protein surface that interacts with HNE is formed not only by residues 15-19 but also by residues 34-40 that are brought close to this primary loop when the protein folds (HUBE74, HUBE75, OAST88). Acting upon this assumption, we changed amino acid

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residues in a second loop of the EpiNE7 protein to find EpiNE7 (SEQ ID NO:48) derivatives having higher affinity for HNE.

The paragraph on page 267, lines 6-20, has been amended as follows:

In the 1TPA complex, ARG<sub>39</sub> contacts SER<sub>96</sub>, ASN<sub>97</sub>, THR<sub>98</sub>, LEU<sub>99</sub> (SEQ ID NO:13), GLN<sub>175</sub>, and TRP<sub>215</sub>. In HNE, all of the corresponding residues are different! SER<sub>96</sub> is deleted; ASN<sub>97</sub> corresponds to ASP<sub>97</sub> (bearing a negative charge); THR<sub>98</sub> corresponds to PRO<sub>98</sub>; LEU<sub>99</sub> corresponds to the residues VAL<sub>99</sub>, ASN<sub>99a</sub>, and LEU<sub>99b</sub>; GLN<sub>175</sub> is deleted; and TRP<sub>215</sub> corresponds to PHE<sub>215</sub>. Position 39 shows a moderately high degree of variability with 7 different amino acids observed, viz. ARG, GLY, LYS, GLN, ASP, PRO, and MET. Having seen PRO (the most rigid amino acid), GLY (the most flexible amino acid), LYS and ASP (basic and acidic amino acids), we assume that all amino acids are structurally compatible with the aprotinin backbone. Because the context of residue 39 has changed so much, we allow all 20 amino acids.

The paragraph on page 270, lines 10-31, has been amended as follows:

Following this second round of affinity selection, a portion of the beads was mixed with XL1-Blue™ cells and plated to allow plaque formation. Of the resulting plaques, 480 were pooled to form a phage population for a third affinity selection. We repeated the selection procedure described above using a population of phage particles containing  $3.0 \cdot 10^9$  plaque forming units. Portions of the pH 2.0 eluate and of the beads were plated with XL1-Blue™ cells to allow formation of plaques. Individual plaques were picked for preparation of RF DNA. From DNA sequencing, we determined the amino acid sequence in the mutated secondary loop of 15 EPiNE7-homolog clones. The sequences are given in Table 210 as EPiNE7.1 through EPiNE7.20 (SEQ ID NOs:59-70). Three sequences were

observed twice: EpiNE7.4 and EpiNE7.14 (SEQ ID NO:63); EpiNE7.8 and EpiNE7.9 (SEQ ID NO:60); and EpiNE7.10 and EpiNE7.20 (SEQ ID NO:65). EpiNE7.4 was eluted at pH 2 while EpiNE7.14 was obtained by culturing HNE beads that had been washed with pH 2 buffer. Similarly, EpiNE7.10 came from pH 2 elution but EpiNE7.20 came from beads. EpiNE7.8 and EpiNE7.9 both came from pH 2 elution. Interestingly, EpiNE7.8 is found in both the first and second fractionations (EpiNE7.31 (vide infra)).

The paragraph on pages 271-272, lines 23-34 and 1-18, has been amended as follows:

In a third round of affinity selection, a population of phage particles containing  $3.0 \cdot 10^{10}$  plaque forming units was added to 20  $\mu$ l of 50% slurry of agarose-immobilized HNE beads and incubated for 2 hours at room temperature. We eluted the phage with the following pH washes: pH 7.0, 6.0, 5.0, 4.5, 4.0, 3.5, 3.25, 3.0, 2.75, 2.5, 2.25, and 2.0. After plating a portion of the pH 2.0 eluate fraction for plaque formation, we picked individual plaques for preparation of RF DNA. DNA sequencing yielded the amino acids sequence in the mutated secondary loop for 20 EpiNE7 homolog clones. These sequences, together with EpiNE7 (SEQ ID NO:48), are given in Table 210 as EpiNE7.21 through EpiNE7.40 (SEQ ID NOS:71 through 87). The plaques observed when EpiNEs are plated display a variety of sizes. EpiNE7.21 through EpiNE7.30 (SEQ ID NOS:71 through 80) were picked with attention to plaque size: 7.21, 7.22, and 7.23 from small plaques, 7.24 through 7.30 from plaques of increasing size, with 7.30 coming from a large plaque. TRP occurs at position 39 in EpiNE7.21, 7.22, 7.23, 7.25, and 7.30. Thus plaque size does not correlate with the appearance of TRP at 39. One sequence, EpiNE7.8, from this fractionation is identical to sequence EpiNE7.8 and EpiNE7.9 obtained in the first fractionation. EpiNE7.30, EpiNE7.34, and EpiNE7.35 are identical, indicating that the diversity of the library has

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been greatly reduced. It is believed that these sequences have an affinity for HNE that is at least comparable to that of EpiNE7 and probably higher. Because the parental EpiNE7 sequence did not recur, it is quite likely that some or all of the EpiNE7-.nn derivatives have higher affinity for HNE than does EpiNE7.

The paragraph on page 272, lines 24-31, has been amended as follows:

Second, the parental sequence has not recurred. At 39, MET did not occur! At position 34 VAL occurred only once in 35 sequences. At 41, ASN occurred only 4 of 35 times. At 40, GLY occurred 17 of 35 times. At position 36, GLY occurred 34 of 35 times, indicating that ALA is undesirable here. EpiNE7.24 (SEQ ID NO:74) and EpiNE7.36 (SEQ ID NO:83) are most like EpiNE7 (SEQ ID NO:48), having three of the varied residues identical to EpiNE7.

The paragraph on pages 273-274, lines 24-34 and 1-28, has been amended as follows:

At position 39, all 20 amino acids were allowed, but only seven were seen. TRP is strongly preferred with 19 occurrences, HIS second with six occurrences, and LEU third with 5 occurrences. No homologues of aprotinin have been reported having either TRP or HIS at position 39 as are now disclosed. Although LEU is represented in the NNS codon thrice, TRP and HIS have but one codon each and their prevalence is surprising. We constructed a model having HNE (Brookhaven Protein Data Bank entry 1HNE) and EpiNE7.9 (SEQ ID NO:60) spatially related as in the 1TPA complex. (The  $\alpha$  carbons of HNE of conserved internal residues were superimposed on the corresponding  $\alpha$  carbons of trypsin, rms deviation  $\approx 0.5$  Å.) Inspection of this model indicates that TRP<sub>39</sub> could interact with the loop of HNE that comprises VAL<sub>99</sub>, ASN<sub>99a</sub>, and LEU<sub>99b</sub>. HIS is observed in six cases; HIS is

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hydrophobic, aromatic, and in some ways similar to TRP. LEU<sub>39</sub> in EpiNE7.5 could also interact with these residues if the loop moves a short distance. GLU occurred twice while LYS, ARG, and GLN occurred one each. In BPTI, the C<sub>α</sub> of residue 39 is ≈10 Å from the C<sub>α</sub> of residue 15 so that TRP<sub>39</sub> interacts with different features of HNE than do the amino acids substituted at position 15. Residue 34 is well separated from each of the residues 15, 18 and 39; thus it contacts different features on the HNE surface from these residues. Although serine proteases are highly similar near the catalytic site, the similarity diminishes rapidly outside this conserved region. The specificity of serine proteases is in fact determined by more interactions than the P1 residue. To make an inhibitor that is highly specific to HNE, we must go beyond matching the requirement at P1. Thus, the substitutions at 19 (determined in Example IV), 39, 34, and other non-P1 positions are invaluable in customizing the EpiNE to HNE. When making an inhibitor customized to a different serine protease, it is likely that many, if not all, of these positions will be changed to obtain high affinity and specificity. It is a major advantage of the present method that many such derivatives may be tested rapidly.

The paragraph on page 275, lines 11-14, has been amended as follows:

There has been little if any enrichment at positions 40 and 41. Alanine is somewhat preferred at 40: ALA:GLY::~~19~~  
~~18:16-17~~. Both ALA and GLY have been reported in aprotinin homologues.

The paragraph on page 275, lines 21-26, has been amended as follows:

One sequence, EpiNE7.25 (SEQ ID NO:75) contains an unexpected change at position 47, SER to LEU. Heretofore, all homologues of aprotinin reported have had either SER or THR at

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position 47. The side groups of SER and THR can form hydrogen bonds to main-chain atoms at the beginning of the short  $\alpha$  helix.

The paragraph on page 275, lines 27-30, has been amended as follows:

The consensus sequence, LYS<sub>34</sub>, GLY<sub>36</sub>, TRP<sub>39</sub>, ALA<sub>40</sub>, LYS<sub>41</sub> was not observed. EpiNE7.23 (SEQ ID NO:73) is quite close, differing only at position 40 where the preferences for ALA is very, very weak.

The paragraph on pages 275-276, lines 31-33 and 1-9, has been amended as follows:

We tested EpiNE7.23 (the sequence closest to consensus) against EpiNE7 (SEQ ID NO:48) on HNE beads. Figure 16 shows the fractionation of strains of phage that display these two EpiNes. Phage that display EpiNE7 are eluted at higher pH than are phage that display EpiNE7.23. Furthermore, more of the EpiNE7.23 phage are retained than of the EpiNE7 phage. Note the peak at pH 2.25 in the EpiNE7.23 elution. This suggests that EpiNE7.23 has a higher affinity for HNE than does EpiNE7. In a similar way, we tested EpiNE7.4 (SEQ ID NO:63) and found that it is not retained on HNE so well as EpiNE7. This is consistent with the fractionation not being complete.

The paragraph on pages 279, lines 7-14, has been amended as follows:

The M13 gene III product contains 'stalk-like' regions as implied by electron micrographic visualization of the bacteriophage (LOPE85). The predicted amino acid sequence of this protein contains repeating motifs, which include:  
glu.gly.gly.gly.ser (EGGGS) (SEQ ID NO:10) seven times  
gly.gly.gly.ser (GGGS) (SEQ ID NO:14) three times  
gly.gly.gly.gly.thr (EGGGT) (SEQ ID NO:15) once.



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The paragraph on pages 279, lines 18-30, has been amended as follows:

Two synthetic oligonucleotides were designed and custom synthesized. GLY is encoded by four codons (GGN); when translated in the opposite direction, these codons give rise to THR, PRO, ALA, and SER. The third base of these codons was picked so that translation of the oligonucleotide in the opposite direction would encode SER. When annealed the synthetic oligonucleotides give the following unit duplex sequence (an EGGGS linker):

```
      E   G   G   G   S (SEQ ID NO:10)
5' C.GAG.GGA.GGA.GGA.TC 3' (SEQ ID NO:100)
3'   TC.CCT.CCT.CCT.AGG.C 5' (SEQ ID NO:101)
      (L) (S) (S) (S) (G) (SEQ ID NO:16)
```

The paragraph on pages 279-280, lines 32-35 and 1-6, has been amended as follows:

The duplex has a common two base pair 5' overhang (GC) at either end of the linker which allows for both the ligation of multiple units and the ability to clone into the unique NARI recognition sequence present in OCV's M13MB48 and Gem MB42. This site is positioned within 1 codon of the DNA encoding the interface. The cloning of an EGGGS linker (SEQ ID NO:10) (or multiple linker) into the vector NarI site destroys this recognition sequence. Insertion of the EGGGS linker in reverse orientation leads to insertion of GSSSL (SEQ ID NO:16) into the fusion protein.

The paragraph on page 280, lines 7-15, has been amended as follows:

Addition of a single EGGGS linker at the NarI site of the gene shown in Table 113 leads to the following gene:

```
79  80  80a 80b 80c 80d 80e 81  82  83  84
   G   G   E   G   G   G   S   A   A   E   G   (SEQ ID NO:17)
-----
```

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GGT.GGC.GAG.GGA.GGA.GGA.TCC.GCC.GCT.GAA.GGT (SEQ ID NO:102)  
-----

The paragraph on page 281, lines 31-32, has been amended as follows:

M13.3X4 (GG)C.GGA.TCC.TCC.TCC.CT(C.GCC) (SEQ ID NO:103)  
gly ser ser ser leu (AA 6-10 of SEQ  
ID NO:11)

The paragraph on page 281, lines 34-35, has been amended as follows:

M13.3X7 (G C.GAG.GGA.GGA.GGA.TC(C.GCC) (SEQ ID NO:104)  
glu gly gly gly ser (SEQ ID NO: 10)

The paragraph on pages 281-282, line 37 and lines 1-5, has been amended as follows:

M13.3X11 (GG)C.GAG.GGA.GGA.GGA.TCC.GGA.TCC.TCC.  
glu gly gly gly ser gly ser ser  
TCC.CTC.GGA.TCC.TCC.TCC.CT(C.GCCC) (SEQ ID  
NO:105)  
ser leu gly ser ser ser leu (SEQ ID NO:18)

The paragraph on pages 316, line 1, has been amended as follows:

--X<sub>1</sub>-X<sub>2</sub>-C-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-C-X<sub>7</sub>-X<sub>8</sub> (SEQ ID NO:19)--  
|

The paragraph on page 319, lines 22-24, has been amended as follows:

--Mini-proteins with two disulfide bonds may be modelled after the  $\alpha$ -conotoxins, e.g., GI, GIA, GII, MI, and SI. These have the following conserved structure (SEQ ID NOs:20-31):--

Page 321, after line 29, insert a new paragraph as follows:

(RVT.VYT.RRS.VHG.VHG.RMG has SEQ ID NO:106).

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The paragraph on page 322, line 32 to page 323, line 5 has been amended as follows:

--The cone snails (Conus) produce venoms (conotoxins) which are 10-30 amino acids in length and exceptionally rich in disulfide bonds. They are therefore archetypal mini-proteins. Novel mini-proteins with three disulfide bonds may be modelled after the  $\mu$ -(GIIIA, GIIIB, BIIIC) or  $\Omega$ -(GVIA, GVIC, GVIIA, GVIIB, MVIIA, MVIIB, etc.) conotoxins. The  $\mu$ -conotoxins have the following conserved structure (SEQ ID NO:32):--

The paragraph on page 323, lines 13-16, has been amended as follows:

--No 3D structure of a  $\mu$ -conotoxin has been published. Hidaka et al. (HIDA90) have established the connectivity of the disulfides. The following diagram depicts geographutoxin I (also known as  $\mu$ -conotoxin GIIIA), whose sequence is SEQ ID NO:33.--

The paragraph on page 324, line 3, has been amended as follows:

--The  $\Omega$ -conotoxins may be represented as follows (SEQ ID NO:34 through 39):--

Page 325, after line 33, insert a new paragraph as follows:

(VYT.VNT.NNK.VWG has SEQ ID NO:107).

The paragraph on page 327, lines 4-9, has been amended as follows:

--Sequences such as  
HIS-ASN-GLY-MET-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-HIS-ASN-GLY-CYS (SEQ ID NO:40)

and

CYS-ASN-GLY-MET-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-HIS-ASN-GLY-HIS (SEQ ID NO:41)

are likely to combine with Cu(II) to form structures as shown

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in the diagram:--

The paragraph on page 327, line 38, has been amended as follows:

For example (SEQ ID NO:42),

The paragraph on pages 329-330, lines 32-33 and 1-2, has been amended as follows:

--One preferred example of a CYS::zinc cross linked mini-protein comprises residues 440 to 461 of the sequence shown in Figure 1 of HARD90. The residues 444 through 456 (SEQ ID NO:43) may be variegated. One such variegation is as follows:--

The paragraph on page 340, lines 6-9, has been amended as follows:

A. Optimized fsX-gfk Codon, Restrained by [D]+[E] = [K]+[R]

	T	C	A	G	
1	.26	.18	.26	.30	<u>f-g</u>
2	.22	.16	.40	.22	<u>x-f</u>
3	.5	.0	.0	.5	<u>s-k</u>

The paragraph on page 349, the first line after the table, has been amended as follows:

--1 BPTI--BPTI (SEQ ID NO:44)--

Page 446, after line 48, insert the new paragraph as follows:

(The amino acids referred to in Table 130 need not be in sequence, but if they are, the sequences all have SEQ ID NO:88).

Page 450, after line 27, insert the new paragraph as follows:

(The amino acids referred to in Table 131 need not be in sequence, but if they are, the sequences all have SEQ ID

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NO: 88).

The paragraph on page 458, lines 1-2, has been amended as follows:

--Table 160: pH Profile of BPTI-III MK phage and ~~Shad 1~~  
EpiNE1 phage binding to Cat G beads.--

The paragraph on page 458, line 4, has been amended as follows:

~~---BPTI-IIIMK---~~ ---BPTI-IIIMK (BPTI has SEQ ID NO:44)---

The paragraph on page 458, line 17, has been amended as follows:

~~---Shad 1---~~ ---EpiNE1 (EpiNE1 has SEQ ID NO:51)---

Page 465, after line 11, insert the following new paragraph:

(SEQ ID NO:45).

For pages 466-471, see enclosed mark-up pages.

)

CLONE		SEQUENCE									
5	IDENTIFIERS										
	EpINE3 (amino-acid: SEQ ID NO:46)										
		1	1	1	1	1	1	2	2		
		3	4	5	6	7	8	9	0	1	
10	3, 9, 16,	P	C	V	G	F	F	S	R	Y	
	17, 18, 19	CCT.TGC.GTC.GGT.TTC.TTC.TCA.CGC.TAT									
		(DNA: SEQ ID NO:109)									
15	EpINE6 (amino-acid: SEQ ID NO:47)										
		1	1	1	1	1	1	2	2		
		3	4	5	6	7	8	9	0	1	
	6	P	C	V	G	F	F	Q	R	Y	
		CCT.TGC.GTC.GGT.TTC.TTC.CAA.CGC.TAT									
20		(DNA: SEQ ID NO:110)									
	EpINE7 (amino-acid: SEQ ID NO:48)										
		1	1	1	1	1	1	2	2		
		3	4	5	6	7	8	9	0	1	
25	7, 13, 14	P	C	V	A	M	F	P	R	Y	
	15, 20	CCT.TGC.GTC.GCT.ATG.TTC.CCA.CGC.TAT									
		(DNA: SEQ ID NO:111)									
30	EpINE4 (amino-acid: SEQ ID NO:49)										
		1	1	1	1	1	1	2	2		
		3	4	5	6	7	8	9	0	1	
	4	P	C	V	A	I	F	P	R	Y	
35		CCT.TGC.GTC.GCT.ATC.TTC.CCA.CGC.TAT									
		(DNA: SEQ ID NO:112)									

5

CLONE		SEQUENCE							
IDENTIFIERS									
10	EpINE8 (amino-acid: SEQ ID NO:50)	1	1	1	1	1	1	2	2
		3	4	5	6	7	8	9	0
	8	P	C	V	A	I	F	K	R
		CCT.TGC.GTC.GCT.ATC.TTC.AAA.CGC.TCT							
15		(DNA: SEQ ID NO:113)							
	EpINE1 (amino-acid: SEQ ID NO:51)	1	1	1	1	1	1	2	2
20		3	4	5	6	7	8	9	0
	1, 10	P	C	I	A	F	F	P	R
	11, 12	CCT.TGC.ATC.GCT.TTC.TTC.CCA.CGC.TAT							
		(DNA: SEQ ID NO:114)							
25	EpINE5 (amino-acid: SEQ ID NO:52)	1	1	1	1	1	1	2	2
		3	4	5	6	7	8	9	0
	5	P	C	I	A	F	F	Q	R
30		CCT.TGC.ATC.GCT.TTC.TTC.CAA.CGC.TAT							
		(DNA: SEQ ID NO:115)							
35	EpINE2 (amin-acid: SEQ ID NO:53)	1	1	1	1	1	1	2	2
		3	4	5	6	7	8	9	0
	2	P	C	I	A	L	F	K	R
		CCT.TGC.ATC.GCT.TTG.TTC.AAA.CGC.TAT							
		(DNA: SEQ ID NO:116)							

Table 209: DNA sequences and predicted amino acid sequences around the P1 region of BPTI analogues selected for binding to Cathepsin G.

5	Clone	P1					
		15	16	17	18	19	
	BPTI	AAA	GCG	CGC	ATC	ATC	
	(SEQ ID NO:44)	LYS	ALA	ARG	ILE	ILE	
10							
	Epic 1 (a)	ATG	GGT	TTC	TCC	AAA	SEQ ID NO:117
	(SEQ ID NO:54)	MET	GLY	PHE	SER	LYS	
15	Epic 7	ATG	GCT	TTG	TTC	AAA	SEQ ID NO:118
	(SEQ ID NO:55)	MET	ALA	LEU	PHE	LYS	
	Epic 8 (b)	TTC	GCT	ATC	ACC	CCA	SEQ ID NO:119
	(SEQ ID NO:56)	PHE	ALA	ILE	THR	PRO	
20							
	Epic 10	ATG	GCT	TTG	TTC	CAA	SEQ ID NO:120
	(SEQ ID NO:57)	MET	ALA	LEU	PHE	GLN	
	Epic 20	ATG	GCT	ATC	TCC	CCA	SEQ ID NO:121
25	(SEQ ID NO:58)	MET	ALA	ILE	SER	PRO	

(a) Clones 11 and 31 also had the identical sequence.

(b) Clone 8 also contained the mutation Tyr 10 to ASN.



Table 210  
 Derivatives of EpiNE7 (SEQ ID NO:48) Obtained  
 by Variegation at positions 34, 36, 39, 40 and 41

5 EpiNE7 (SEQ ID NO:48)  
 ++++++ \*\*\*\*\*  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFVYGGCmgngNNFKSAEDCMRTCGGA  
 1 2 3 4 5  
 10 1234567890123456789012345678901234567890123456789012345678

↓ ↓ ↓ ↓ ↓      \* \* \* \* ↓  
 15 EpiNE7.6 (SEQ ID NO:59)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFlyGCKgkGNNFKSAEDCMRTCGGA

EpiNE7.8, EpiNE7.9, and EpiNE7.31 (SEQ ID NO:60)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFeyGCwakGNNFKSAEDCMRTCGGA

EpiNE7.11 (SEQ ID NO:61)  
 20 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFgYgCrakGNNFKSAEDCMRTCGGA

EpiNE7.7 (SEQ ID NO:62)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFeyGChaeGNNFKSAEDCMRTCGGA

25 EpiNE7.4 and EpiNE7.14 (SEQ ID NO:63)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFlyGCwaqGNNFKSAEDCMRTCGGA

EpiNE7.5 (SEQ ID NO:64)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFrYgGClaeGNNFKSAEDCMRTCGGA

30 EpiNE7.10 and EpiNE7.20 (SEQ ID NO:65)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFdYgGChadGNNFKSAEDCMRTCGGA

EpiNE7.1 (SEQ ID NO:66)  
 35 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFkyGClahGNNFKSAEDCMRTCGGA

EpiNE7.16 (SEQ ID NO:67)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFtYgGCwanGNNFKSAEDCMRTCGGA

40 EpiNE7.19 (SEQ ID NO:68)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFnYgGCegkGNNFKSAEDCMRTCGGA

EpiNE7.12 (SEQ ID NO:69)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFqYgGCegyGNNFKSAEDCMRTCGGA

45 EpiNE7.17 (SEQ ID NO:70)  
 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFqYgGClgeGNNFKSAEDCMRTCGGA

EpiNE7.21 (SEQ ID NO:71)  
 50 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFhYgGCwgqGNNFKSAEDCMRTCGGA

Table 210: Derivatives of EpiNE7 (SEQ ID NO:48) Obtained  
by Variegation at positions 34, 36, 39, 40 and 41  
(continued)

```

5      *****
EpiNE7 (SEQ ID NO:48)
RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFVYGGCmgngNNFKSAEDCMRTCGGA
      1         2         3         4         5
123456789012345678901234567890123456789012345678

10      ↓↓↓↓          ♦ ♦ ♦ ↓
EpiNE7.22 (SEQ ID NO:72)
RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFhYgGCwgeGNNFKSAEDCMRTCGGA

15      EpiNE7.23 (SEQ ID NO:73)
RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFkYgGCwggkGNNFKSAEDCMRTCGGA

      EpiNE7.24 (SEQ ID NO:74)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFkYgGChgnGNNFKSAEDCMRTCGGA

20      EpiNE7.25 (SEQ ID NO:75)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFpYgGCwakGNNFKLAEDCMRTCGGA

      EpiNE7.26 (SEQ ID NO:76)
25      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFkYgGCwghGNNFKSAEDCMRTCGGA

      EpiNE7.27 (SEQ ID NO:77)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFnYgGCwggkGNNFKSAEDCMRTCGGA

30      EpiNE7.28 (SEQ ID NO:78)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFtYgGClghGNNFKSAEDCMRTCGGA

      EpiNE7.29 (SEQ ID NO:79)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFtYgGClgyGNNFKSAEDCMRTCGGA

35      EpiNE7.30, EpiNE7.34, and EpiNE7.35 (SEQ ID NO:80)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFkYgGCwaeGNNFKSAEDCMRTCGGA

      EpiNE7.32 (SEQ ID NO:81)
40      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFgYgGCwgeGNNFKSAEDCMRTCGGA

      EpiNE7.33 (SEQ ID NO:82)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFeYgGCwanGNNFKSAEDCMRTCGGA

45      EpiNE7.36 (SEQ ID NO:83)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFvYgGChgdGNNFKSAEDCMRTCGGA

      EpiNE7.37 (SEQ ID NO:84)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFmYgGCqgkGNNFKSAEDCMRTCGGA

50      EpiNE7.38 (SEQ ID NO:85)
      RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFyYgGCwakGNNFKSAEDCMRTCGGA

```

Table 210 (continued)  
 Derivatives of EpINE7 (SEQ ID NO:48) Obtained  
 by Variegation at positions 34, 36, 39, 40 and 41

5

EpINE7 (SEQ ID NO:48)

♦♦♦♦♦

\*\*\*\*\*

 RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFVYGGCmgngNNFKSAEDCMRTCGGA  
 1 2 3 4 5

101234567890123456789012345678901234567890123456789012345678

↓ ↓ ↓ ↓ ↓

♦ ♦ ♦ ♦ ↓

EpINE7.39 (SEQ ID NO:86)

15RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFmYgGCwgdGNNFKSAEDCMRTCGGA

EpINE7.40 (SEQ ID NO:87)

RPDFCLEPPYTGPCvAmfpRYFYNAKAGLCQTFtYgGChgnGNNFKSAEDCMRTCGGA

Table 210: Derivatives of EpiNE7 Obtained  
by Variegation at positions 34, 36, 39, 40 and 41  
(continued)

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## Notes:

- a) † indicates variegated residue. \* indicates imposed change. ↓ indicates carry over from EpiNE7.
- 10 b) The sequence M<sub>39</sub>-GNG in EpiNE7 (indicated by \*) was imposed to increase similarity to ITI-D1.
- 15 b) Lower case letters in EpiNE7.6 to 7.38 indicate changes from BPTI, that were selected in the first round (residues 15-19) or positions where the PBD was variegated in the second round (residues 34, 36, 39, 40, and 41).
- 20 c) All EpiNE7 derivatives have G<sub>42</sub>.